Reference: *Biol. Bull.* **166**: 328–348. (April, 1984)

BIOMETRICAL AND HISTOLOGICAL ASPECTS OF THE REPRODUCTIVE CYCLE OF THE OVARIES OF *ASTERIAS RUBENS* (ECHINODERMATA)

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Abstract

The reproductive cycle of the ovaries of the starfish *Asterias rubens* has been studied by histological and biometrical methods and can be divided into 4 stages, based on semi-quantitative and biometrical data. On the basis of biometrical data and their statistical analyses, each stage can be subdivided into two substages.

Frequency distributions of oocyte diameters present a characteristic for each (sub)stage. McCall units were calculated from the frequency distribution of oocyte diameters of stage 3. This stage is the reference for each other stage or substage and can be used as reference for any other group of animals of the species under examination. McCall transformations allow for the comparison of frequency distributions. It is concluded that the oocyte diameter is a reliable parameter for delineating the reproductive cycle and oogenesis of *Asterias rubens*, but a detailed description of the annual reproductive cycle required the analysis of a complex of variables, including the correlations among them.

From the stages' frequency distributions, the theoretical and proportional duration of each stage and of the period of spawning was derived. Minor, not significant variations have been found from year to year in the duration of the stages and in the beginning and conclusion of each period.

Introduction

Recently, steroidogenesis of ovaries and pyloric caeca of the starfish has been studied with some different steroids as precursor (Schoenmakers, 1979; Schoenmakers and Voogt, 1980, 1981). The steroid synthesizing capacity (Schoenmakers, 1980), and the presence and levels of endogenous steroids (Dieleman and Schoenmakers, 1979; Schoenmakers and Dieleman, 1981) have been determined during the reproductive cycle. Information on changes of the ovaries during the reproductive cycle will lead to a better understanding of these biochemical data.

Schoenmakers *et al.* (1981b) described the histology and ultrastructure of the ovaries of the starfish *Asterias rubens* and divided oogenesis into four phases: a) multiplication phase of oogonia, b) initial growth phase of oocytes I, c) growth phase proper of oocytes I, and d) post-growth phase of oocytes I. They also observed that the oocytes within an animal are seldom all in the same phase of oogenesis.

In several studies on the reproduction of echinoderms the question has been raised as to which criteria are the most appropriate for delineating the reproductive cycle. A number of studies on echinoderm reproductive cycles are based on the gonad index (Lasker and Giese, 1954) for measuring reproductive conditions (Feder, 1956; Farmanfarmaian *et al.*, 1958; Greenfield, 1959; Boolootian, 1966). Patent (1969), however,

Received 27 May 1983; accepted 16 December 1983.

pointed out that the gonad index may give a useful measure of the relative size of the gonads, but fails to indicate the actual condition of the gametes. Moreover, the gonads of some echinoderms, such as *Gorgonocephalus caryi,* cannot be removed in total, so that a gonad index cannot be determined (Patent, 1969). Other authors applied periodic histological examinations of the gonads as a means of determining the reproductive cycle in echinoderms (Yoshida, 1952; Tanaka, 1958). Chia (1968) studied the cyclical changes of the oocytes of *Leptasterias hexactis* by measuring the diameter of both fresh and fixed material on a monthly basis. Pearse (1965) showed the oocyte changes of *Odontaster validus* with frequency polygons, averaged for all females of each sample. Patent (1969) measured the diameter of twenty of the largest oocytes from each female specimen and determined the mean. For *Asterias rubens* Vevers (1949) measured the length of the ovaries and the diameter of the oocytes; Kowalski (1955) determined the gonad index and the diameter of fresh oocytes, and Jangoux and Vloebergh (1973) composed frequency polygons of oocyte diameters and measured the gonad index.

Applying histological and biometrical methods, the present study deals with the analysis of a complex of variables, with which the reproductive cycle of *Asterias rubens* can be divided into stages and substages.

Materials and Methods

Animals

Asterias rubens were collected from the Wadden Sea, east of the island of Texel (The Netherlands), at three week intervals during 2.5 years. The animals were kept in aerated sea water at 6°C for three days until processing. Ovaries of five females from each sample were studied with the light microscope. Only specimens with arms varying from 6.0 to 10.0 cm were used, the length measured from the center of the mouth along the oral face to the tip of the arm. Parasitized (parasite: *Orchitophyra stellarum*) animals were discarded.

Data on the course of quantitative parameters during the reproductive cycle, and semi-quantitative data were obtained from animals collected during 1 year (n = 83). The approximation of the duration of the stages of the reproductive cycle was based on data from all the collected animals (n = 188).

Dissection of animals

After cutting the arms laterally on both sides, the gonads were separated from the gonoduct. The animals were sexed by means of a squash preparation of gonadal tissue, which was also examined for the possible presence of parasites.

Histological procedure

Small pieces from the proximal, the middle, and the distal part of the ovaries were fixed in Bouin's fluid, embedded in paraffin, and sectioned at 7 μm. Sections were stained with Brookes' trichrome (Brookes, 1968) and Periodic Acid Schiff-Orange G (PAS/OG). Pilot studies showed the structure to be the same in the different parts of the ovaries. For this reason, detailed examinations were made only in cross-sections of the middle part of the ovaries.

In the Brookes-stained sections the percentages of pear-shaped oocytes and basophilic oocytes were determined and the presence or absence of the following characteristics was scored for each animal: dividing oogonia in the germinal epithelium,

cell-debris, phagocytozing cells, follicle cells, jelly coat, cortical vesicles, degenerating oocytes, and "loose" oocytes. In the PAS/OG-stained sections the percentage of PAS-positive oocytes was determined and the presence or absence of invaginations of the hemal system and the amount of the hemal fluid was scored for each animal. The semi-quantitative scores have the following gradation: − = absent; ± = scarce; + = present; ++ = frequent; +++ = abundant.

Histometric procedure and processing of data

To obtain morphometric data on the variation of oocytes, the Brookes-stained sections of ovaries of each animal were used. Representative areas, containing at least 100 oocytes, were traced from the ovarian wall to the middle of the ovarian lumen, and drawn with a Zeiss microscope, equipped with camera lucida. The area of each of these oocytes was measured with a digitizer (Hewlett-Packard, type 9864A) in combination with a calculator (Hewlett-Packard, type 9820). Approximating the sectioned oocytes as circles, the diameters of these oocytes were calculated, averaged, and the 95% confidence interval (CI) was determined. Also the average (with CI) of the ten smallest oocyte diameters and of the ten largest oocyte diameters were computed for each animal. Finally, the median value of one hundred oocytes was determined for each animal.

These quantitative and also the semi-quantitative data were used for dividing the reproductive cycle into stages. First of all, the oocyte diameters of every single animal were compared with those of all other animals. For that purpose the one hundred oocyte diameters of one animal were pooled with those of another animal and arranged in order of ascending size to determine the common median. The presence or absence of differences between the two animals was then statistically analyzed by applying the median test for the 2×2 contingency table, according to Sokal and Rohlf (1969). This procedure was carried out for all animals. The specimens not showing significant differences in the oocyte diameters were placed in groups or stages. For each stage the frequency distribution of the oocyte diameter was determined. The symmetry of each distribution was checked, using the symmetry test (Scharf *et al.*, 1968). In case of asymmetry ($\chi^2 > 3.841$) McCall (1949) transformations were applied according to Scharf *et al.* (1968). The McCall units (T-values) were calculated from the frequency distribution of oocyte diameters of the stage that contains, with a range of 7–140 μm, almost all the possible diameters found in all stages or substages together. This frequency distribution was used as the reference, so that the calculated T-values, corresponding with diameters between 7 and 140 μm, could also be applied to corresponding diameters of each other stage or substage. The other T-values were found by interpolation or extrapolation via quadratic regression. The significance of differences between the transformed frequency distributions was determined by using the excentricity test. The oocyte diameters of each stage were also plotted as cumulative quantile, and next the semi-interquartile range was calculated. In addition, the other parameters were used to check this subdivision of the reproductive cycle. Therefore, the significance of differences between the data for the stages of this subdivision was determined for each parameter with the Student's *t*-test and the test of Wilcoxon for quantitative and semi-quantitative data, respectively.

Based on a comparison of the semi-quantitative data with the quantitative results and their statistical evaluations, it was possible to halve the number of stages by combining two subsequent stages at a time, starting from spawning. Thus the reproductive cycle can be divided into a number of stages, and each stage into two substages. For each stage, *i.e.*, combination of two substages, the frequency distribution and the

cumulative quantile of the oocyte diameters were plotted and the semi-interquartile range was calculated. Following these procedures, the stage of the reproductive cycle is obtained for each animal separately. This made it possible to determine the maturation index, *i.e.*, the average stage per sample. Moreover, the proportional distribution of the stages per sample can be determined and this produces the frequency distribution of each stage. From the mean and standard deviation of the frequency distribution of the stages, the theoretical duration of the stages can be approximated, using 3× the standard deviation, right and left of the mean. The actual number of days of one reproductive cycle was defined as the number of days between the mean of the frequency distribution of a certain stage in one reproductive cycle and the mean of the frequency distribution of that stage in the following reproductive cycle. The ratio between this number of days and the sum of the theoretical durations of all stages of one reproductive cycle can be calculated. The proportional (relative) duration of each stage was computed from the theoretical duration by multiplying it with this ratio. Then, the excentricity of the proportional duration, its corresponding proportional area of the frequency distribution, and its tail-probability are to be calculated.

The course of quantitatively determined variables during the reproductive cycle also was studied. For that purpose the mean percentage (±SEM) per sample of pear-shaped oocytes and of the PAS-positivity and basophilia of the oocytes, the mean (±CI) per sample of the pyloric caeca index and of the gonad index, the weighed average per sample of the ten smallest, of the ten largest, and of one hundred oocyte diameters, and the mean (±SEM) per sample of the median values were calculated. These data (and the maturation indices) were plotted *versus* time. Correlation between the quantitatively determined variables was examined for the individual data using linear regression by the method of least squares and the correlation test.

Calculations and statistics

The diameters were calculated from the areas of the oocytes, approximating the sectioned oocytes as circles. The standard deviations (S.D.), the standard error of the mean (SEM) and the 95% confidence interval (CI) were calculated as usual.

The weighed averages of the average values for the animals per sample were determined with the formula:

$$\bar{x}_w = \frac{\sum w_i \times x_i}{\sum w_i} \pm \frac{1}{\sqrt{\sum w_i}}, \quad (\bar{x}_w = \text{weighed average}; \; w_i = \frac{1}{(CI)^2}).$$

The maturation index was calculated according to Yoshida (1952) as:

$$MI = \frac{\sum (n \times F)}{N}, \quad (n = \text{number of animals in stages F};$$

$$N = \text{total number of animals in the sample}).$$

The organ index was expressed as the percentage organ weight (fresh) of the total weight.

The semi-interquartile range (Q) was calculated with the formula:

$$Q = \frac{Q_3 - Q_1}{2}, \quad (Q_1 = \text{value, below which 25\% of the values;}$$

$$Q_3 = \text{value, below which 75\% of the values}).$$

The median test for the 2×2 contingency table was performed using the equation:

$$\chi^2 = \frac{n(ad-bc)^2}{(a+b)(c+d)(a+c)(b+d)}$$ (equation 16.9, Sokal and Rohlf, 1969).

The differences between the oocyte diameters of two animals are not significantly different, if $\chi^2 < 3.841$ ($P = 0.05$).

The symmetry of the frequency distribution of oocyte diameters of each stage was tested according to Scharf *et al.* (1968) with the formula:

$$\chi^2 = \frac{(H^+ + H^-)^2}{n}$$

in which:

$H^+ = \sum y(x > \bar{x})$ = number of oocyte diameters larger than the mean

$H^- = \sum y(x < \bar{x})$ = number of oocyte diameters smaller than the mean

$n = \sum y$ = total number of oocytes diameters.

A frequency distribution is concluded to be asymmetric, if $\chi^2 > 3.841$ ($P = 0.05$).

McCall transformation

The method of transformation (McCall, 1949) is based on the determination of a correction factor for the abscissa. To that end ζ_m is calculated via the frequency of oocyte diameter (y_m) with the following formulas:

$$\eta_m = 0.5y_m + \sum_{N}^{m+1} y_i,$$

($i = 1, 2 \cdots N$; $m = 0, 1, 2 \cdots N$; N = number of frequencies).

$$\zeta_m = \frac{\eta_m + 100(\%)}{n}, \quad (n = \sum y = \text{total number of oocyte diameters}).$$

The McCall value T_m can be found in a table via ζ_m. Using a computer the McCall value T_m can be calculated via a function. Firstly, the factor u_m must be calculated via the formula:

$$u_m = \frac{\zeta_m - 1}{50}$$

T_m can be computed by using the function (the indices have been omitted):

$$T = a_1 - a_2 \cdot \text{arctanh}(u) + a_3 \cdot \text{arAmp}(u) + a_4 \cdot \tan(u) + a_5 \cdot \sinh(u) - a_6 \cdot \arcsin(u) + a_7 \cdot (u)^3,$$

in which:

$a_1 = 49.99893727$ $\quad$ $a_5 = 639.378889$

$a_2 = 4.32564762$ $\quad$ $a_6 = 28.6845627$

$a_3 = 827.489609$ $\quad$ $a_7 = 34.2207938$

$a_4 = 208.528568$

RESULTS

By using the median test, statistical analysis of the oocyte diameters produced eight significantly different groups of animals. The animals belonging to a certain group are not significantly different from each other ($\chi^2 < 3.841$). On the basis of these results the annual reproductive cycle of *Asterias rubens* can be subdivided into eight substages. Essential data of the frequency distributions of the oocyte diameters for these substages before and after McCall (1949) transformation are summarized in Table I. Differences between them appear to be significant in all cases ($P < 0.0001$). This confirms the subdivision of the reproductive cycle into eight substages. Based on this subdivision, the significance of differences between the data for the substages was determined for each parameter, using the Student's *t*-test or the test of Wilcoxon. The median values and the averages of the one hundred oocytes diameters also permit us to distinguish between eight substages ($P < 0.005$; Fig. 1). However, a comparison of these results with the other parameters, especially the combination of the semi-quantitative data, provides arguments in support of reducing the eight substages to four stages, by combining two subsequent substages at a time, starting from spawning. Consequently, the annual reproductive cycle can be divided into four stages, each of which can be subdivided into two substages, on the basis of the biometrical results.

As to the other quantitative data, the averages of the ten largest oocytes determine the substages 1a and 1b, 2a and 2b, and 3a and 3b ($P < 0.005$), the averages of the ten smallest oocytes the substages 3a and 3b, and 4a and 4b ($P < 0.005$); the percentage

TABLE I

The oocyte diameters of specimens of Asterias rubens *in the eight substages and four stages of the annual reproductive cycle*

Substage	Number of specimens	Oocyte diameter (μm)	Symmetry[1]	Transformed oocyte diameter (McCall units)	Symmetry[2]
1a	14	23.31 ± 0.19	−	29.28 ± 0.15	+
1b	12	27.16 ± 0.21	−	32.07 ± 0.15	−
2a	7	36.33 ± 0.41	+	37.13 ± 0.19	− ($\chi_x^2 < \chi_T^2$)
2b	7	42.23 ± 0.49	+	39.62 ± 0.23	+
3a	13	60.20 ± 0.62	+	47.07 ± 0.25	+
3b	12	74.52 ± 0.65	+	52.81 ± 0.26	+
4a	10	92.95 ± 0.70	−	60.25 ± 0.28	− ($\chi_x^2 > \chi_T^2$)
4b	8	102.02 ± 0.56	−	63.93 ± 0.24	+
Stage					
1	26	25.23 ± 0.15	−	30.67 ± 0.11	+
2	14	39.72 ± 0.37	−	38.53 ± 0.18	− ($\chi_x^2 > \chi_T^2$)
3	25	67.51 ± 0.49	+	50.00 ± 0.20[3]	+
4	18	97.55 ± 0.49	−	62.13 ± 0.20[4]	− ($\chi_x^2 > \chi_T^2$)

[1] Symmetry of the frequency distribution of oocyte diameters before McCall transformation.

[2] Symmetry of the frequency distribution of oocyte diameters after McCall transformation.

[3] The McCall units (T-values) were calculated from the frequency distribution of oocyte diameters of stage 3. Since the frequency distribution, with a range of 7–140 μm, contains nearly all the possible oocyte diameters found in all stages or substages together, it is used as reference for each other stage or substage. Therefore, the calculated T-values, corresponding with diameters between 7 and 140 μm, are also applied to corresponding diameters of each other stage or substage.

[4] Results expressed as means ± SEM.

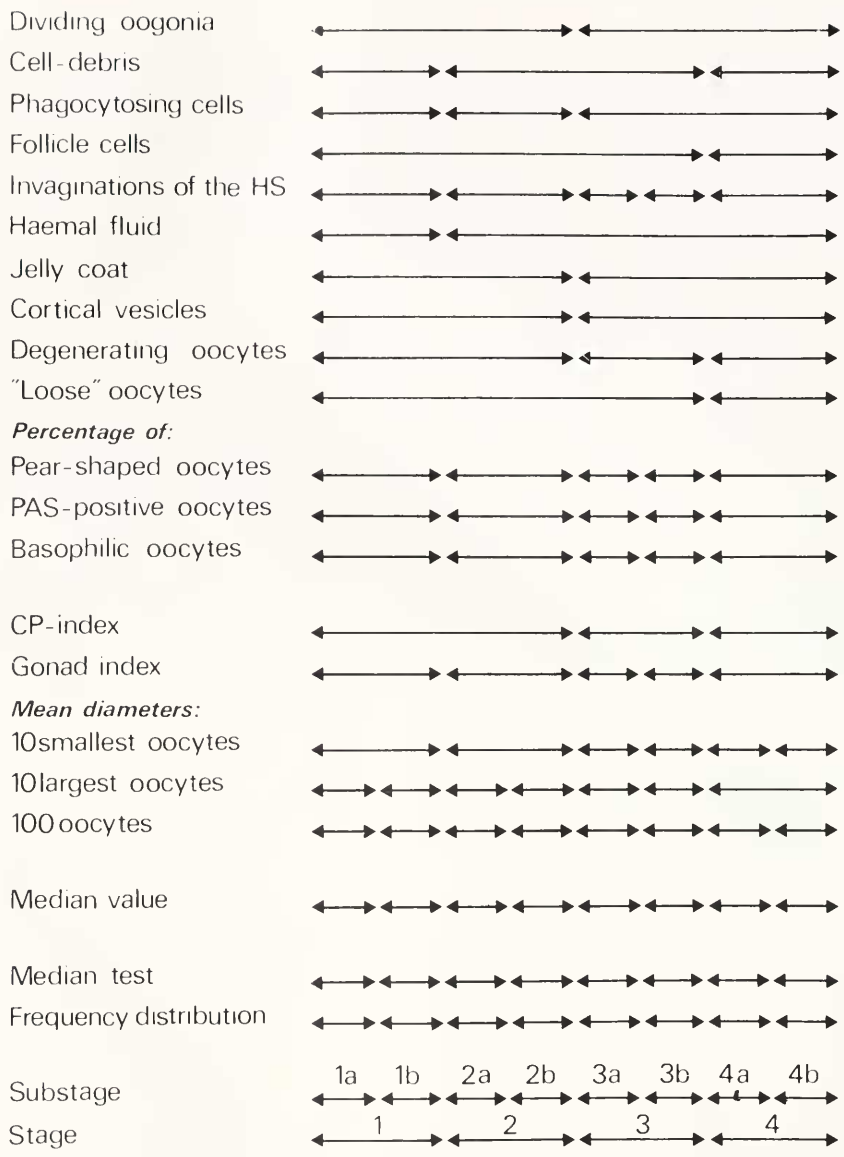

FIGURE 1. Summary of parameters and for each an outline of significant changes, indicated by arrows, from (sub)stage to (sub)stage during the annual reproductive cycle of *Asterias rubens*. HS: hemal system; CP: pyloric caeca.

of pear-shaped oocytes, the percentages of PAS-positivity and basophilia of oocytes, and the gonad index together define the substages 3a and 3b ($P < 0.005$).

Dividing oogonia in the germinal epithelium, cell-debris, phagocytozing cells, and follicle cells are shown in Plate I. Invaginations of the hemal system are depicted in Plate II, 1 and 2, the hemal fluid in Plate II, 3 and 4. The jelly coat and cortical vesicles of full-grown oocytes are visible in Plate III, 1 and 2, respectively. Examples

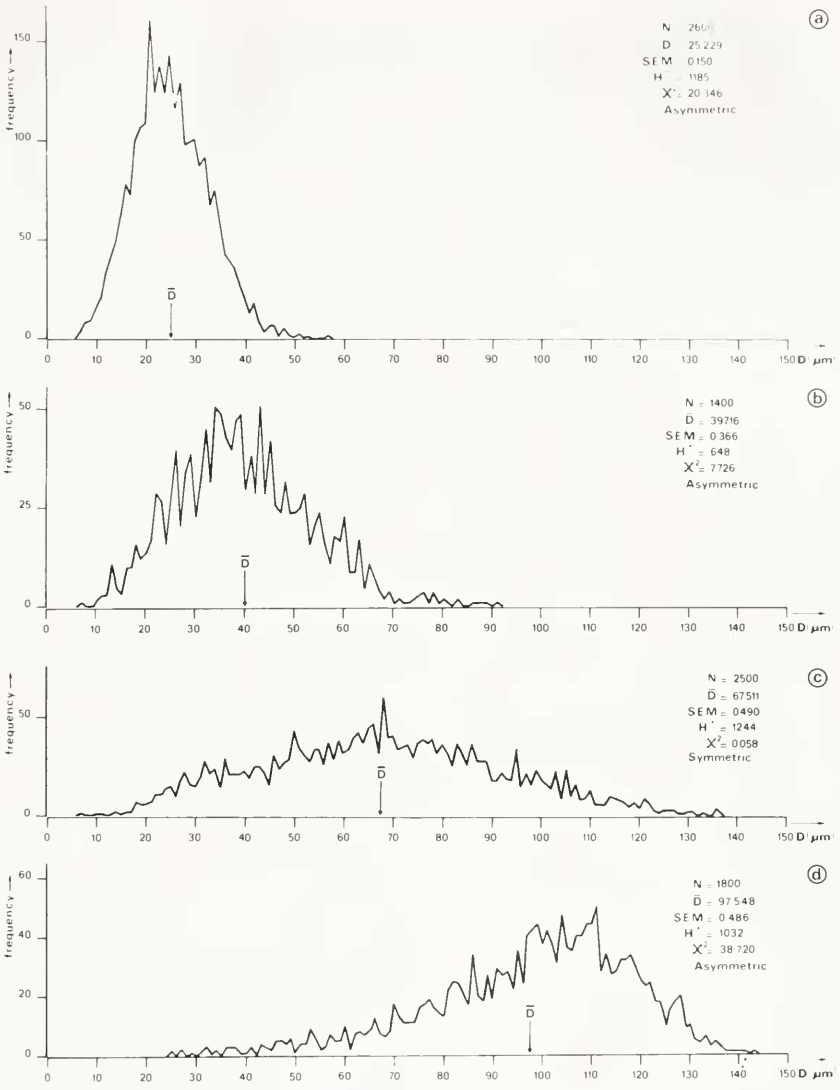

FIGURE 2. Frequency distributions of oocyte diameters of *Asterias rubens* (before McCall transformation). a: For the resting stage (stage 1; substages 1a and 1b); b: for the early growth stage (stage 2; substages 2a and 2b); c: for the stage of late growth and partial maturation (stage 3; substages 3a and 3b); d: for the maturation stage (stage 4; substages 4a and 4b).

of degenerating and "loose" oocytes are presented in Plate III, 3 and 4. All these parameters have been semi-quantitatively scored for each animal. The semi-quantitative data, obtained in this manner, do not support a subdivision into eight substages. The individual semi-quantitatively determined parameters hardly justify four stages. Only the decrease in the number of invaginations of the hemal system shows four stages ($P < 0.025$) and permits stage 3 to be subdivided into two substages ($P < 0.05$). The absence of dividing oogonia and the presence of the jelly coat, cortical vesicles and degenerating oocytes define stages 3 and 4 ($P < 0.025$, $P < 0.005$, $P < 0.005$, P

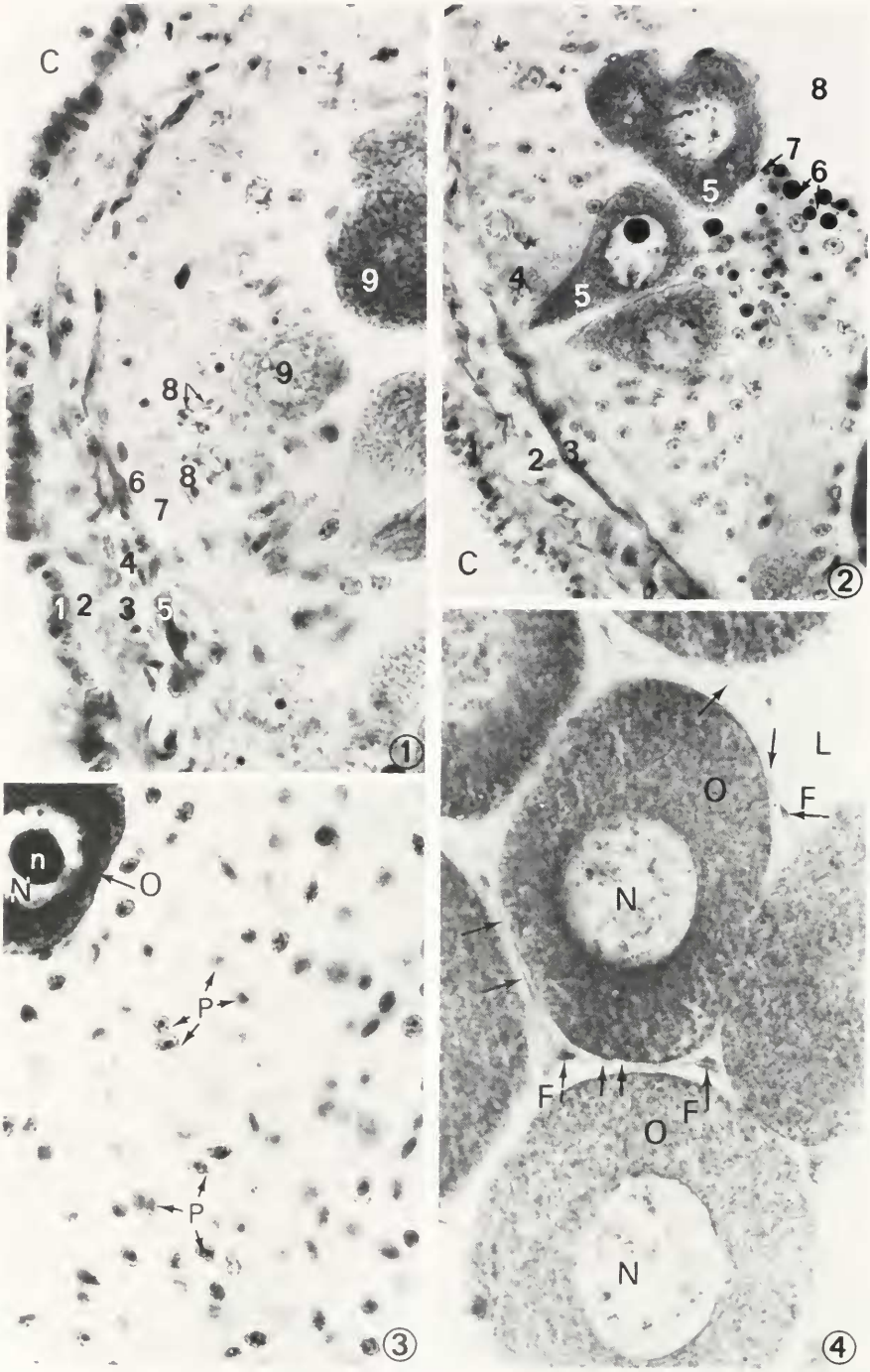

PLATE I: 1. Detail of an ovarian wall of *Asterias rubens*. Brookes' trichrome staining. ×820. C: Coelom; 1: perivisceral coelomic epithelium; 2: connective tissue; 3: layer with outer genital coelomic epithelium and muscle cells; 4: genital coelomic sinus; 5: layer with inner genital coelomic epithelium and muscle cells; 6: hemal system; 7: germinal epithelium; 8: dividing oogonia; 9: oocytes.

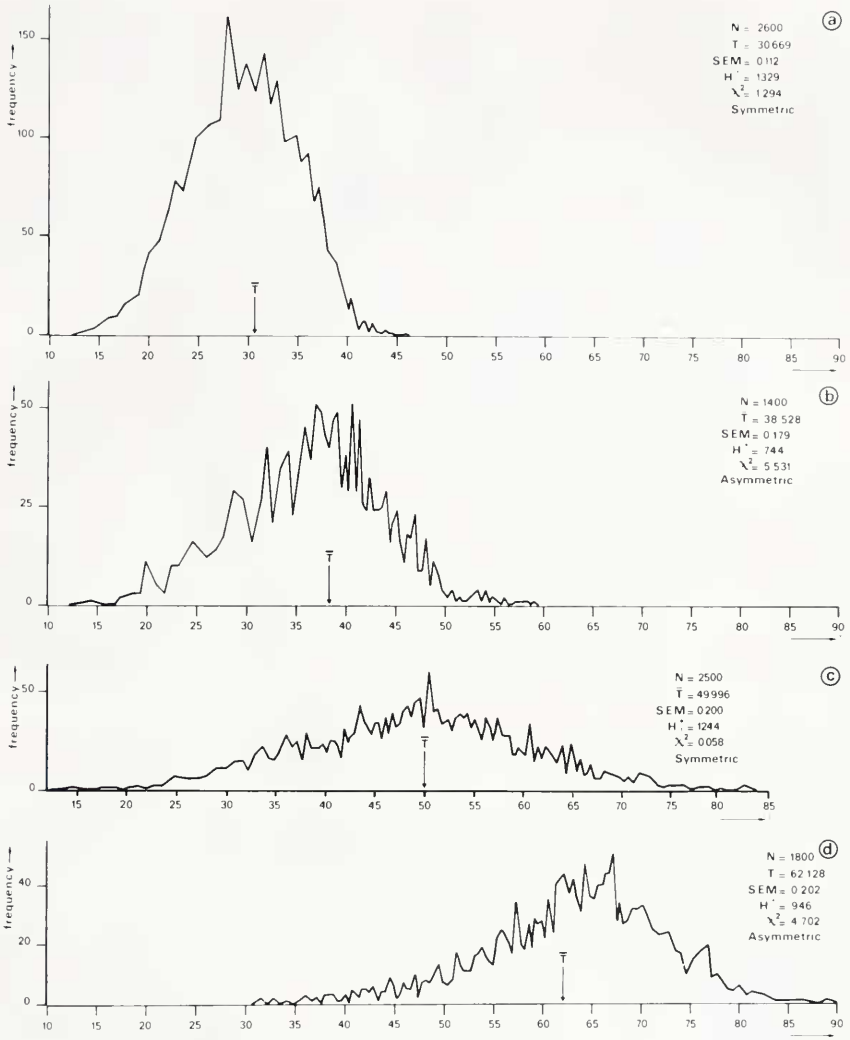

FIGURE 3. Frequency distributions of oocyte diameters of *Asterias rubens* (after McCall transformation). a: for the resting stage (stage 1; substages 1a and 1b); b: for the early growth stage (stage 2; substages 2a and 2b); c: for the stage of late growth and partial maturation (stage 3; substages 3a and 3b); d: for the maturation stage (stage 4; substages 4a and 4b).

< 0.005, respectively) and the increase of degenerating oocytes distinguishes stage 3 from stage 4 ($P < 0.05$). The amount of cell-debris separates stage 1 and stage 4 from the others ($P < 0.005$, $P < 0.005$, respectively); the number of phagocytozing cells

2. Detail of an ovary. Brookes' trichrome staining. ×520. C: Coelom; 1: outer part of the ovarian wall; 2: genital coelomic sinus; 3: inner part of the ovarian wall; 4: germinal epithelium; 5: oocytes; 6: cell-debris; 7: follicle cells; 8: ovarian lumen.

3. Detail of an ovarian lumen. Brookes' trichrome staining. ×820. P: Phagocytozing cells; O: oocyte; N: nucleus; n: nucleolus.

4. Detail of an ovarian lumen. Brookes' trichrome staining. ×520. L: Ovarian lumen; O: oocytes, surrounded by follicle cells (F); N: nucleus. Note the long cellular processes (arrows) of the follicle cells around the oocytes.

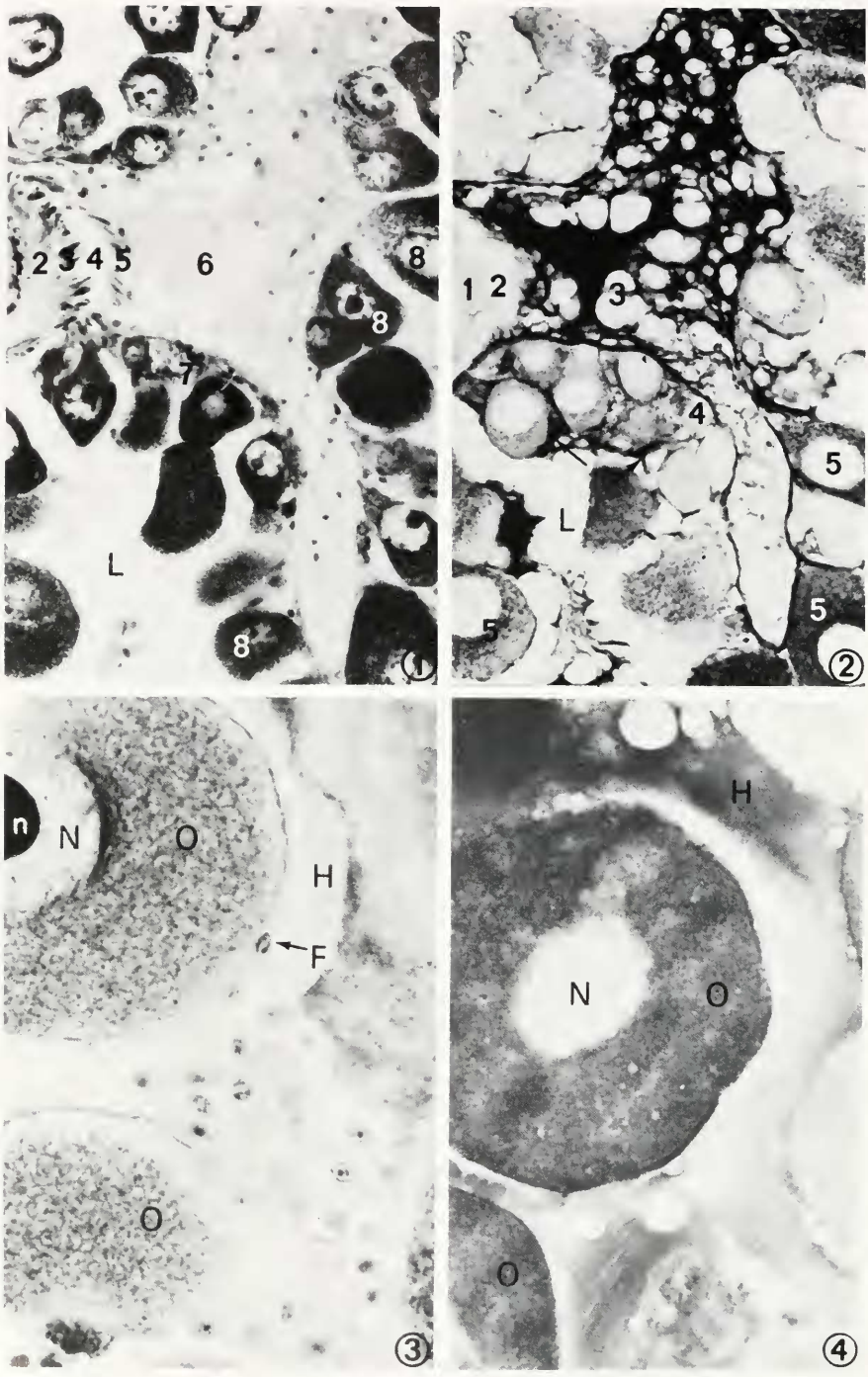

PLATE II: 1. Detail of an ovary of *Asterias rubens*. Brookes' trichrome staining. ×325. 1: Epithelium; 2: connective tissue; 3: layer with epithelium and muscle cells; 4: genital coelomic sinus; 5: layer with epithelium and muscle cells; 6: hemal system with invaginations into the ovarian lumen (L); 7: germinal epithelium; 8: oocytes. Note the close contact between the hemal system and the oocytes.

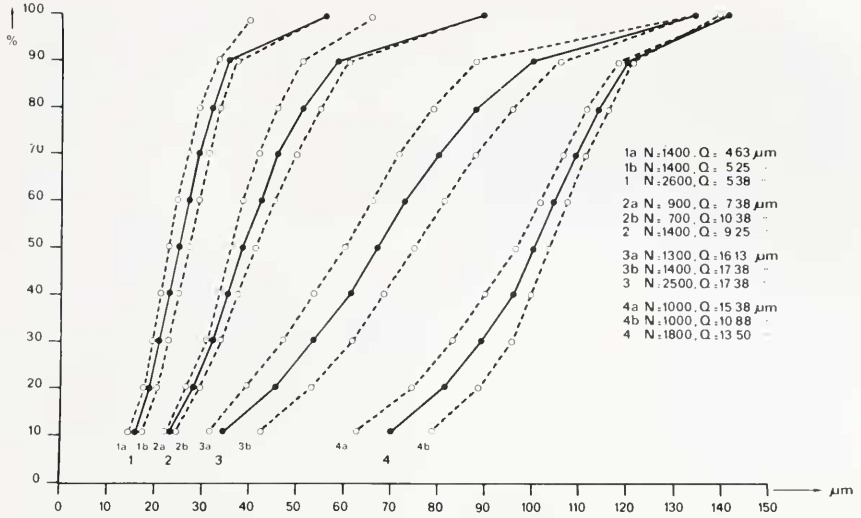

FIGURE 4. Cumulative quantiles of oocyte diameters for the stages and substages of the annual reproductive cycle of *Asterias rubens*. O - - - O: Cumulative quantiles of oocyte diameters from animals belonging to a substage, mutually not significantly different ($\chi^2 < 3.841$). ● —— ●: Cumulative quantiles of oocyte diameters from animals belonging to two subsequent substages, and in the same stage of the annual reproductive cycle. Q: Semi-interquartile range.

distinguishes stage 1 from stage 2 ($P < 0.005$), and stage 2 from stages 3 and 4 ($P < 0.025$). The absence of the hemal fluid in stage 1 distinguishes this stage from the others ($P < 0.005$). Finally, the desintegration of follicle cells and the presence of "loose" oocytes indicates a difference between stage 4 and the other stages ($P < 0.005$, $P < 0.005$, respectively).

For the four stages, obtained in this manner, the frequency distributions of oocyte diameters before and after McCall (1949) transformation are given in Figures 2 and 3, respectively. Essential data of these frequency distributions are summarized in Table I. Differences between these distributions are significant ($P < 0.0001$). For each stage and each substage the cumulative quantile of the oocyte diameters has been plotted (Fig. 4).

Determining the proportional distribution of the stages per sample produces the frequency distributions of the stages of the reproductive cycle, presented in Figure 5. Some of the frequency distributions are slightly but significantly asymmetric. In approximating the duration of the stages, this asymmetry is neglected. Differences between the frequency distributions of the stages within one reproductive cycle were found to be significant ($P < 0.0001$). The frequency distributions of the stages of one reproductive cycle are different from those of the following cycle, but the differences proved to be not significant. The frequency distribution of each stage and the dis-

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2. Parallel section to Part I. PAS/OG staining. ×325. 1: Outer part of the ovarian wall; 2: genital coelomic sinus; 3: the hemal system, PAS-positive, with invaginations into the ovarian lumen (L), branching retiformly between the oocytes (arrows); 4: germinal epithelium; 5: oocytes.

3. Detail of an ovarian lumen. Brookes' trichrome staining. ×820. O: Oocytes; N: nucleus; n: nucleolus; F: follicle cell; H: hemal fluid between the oocytes.

4. Detail of an ovarian lumen. PAS/OG staining. ×520. O: Oocytes; N: nucleus; H: hemal fluid, a mucoid-like and PAS-positive substance between the oocytes.

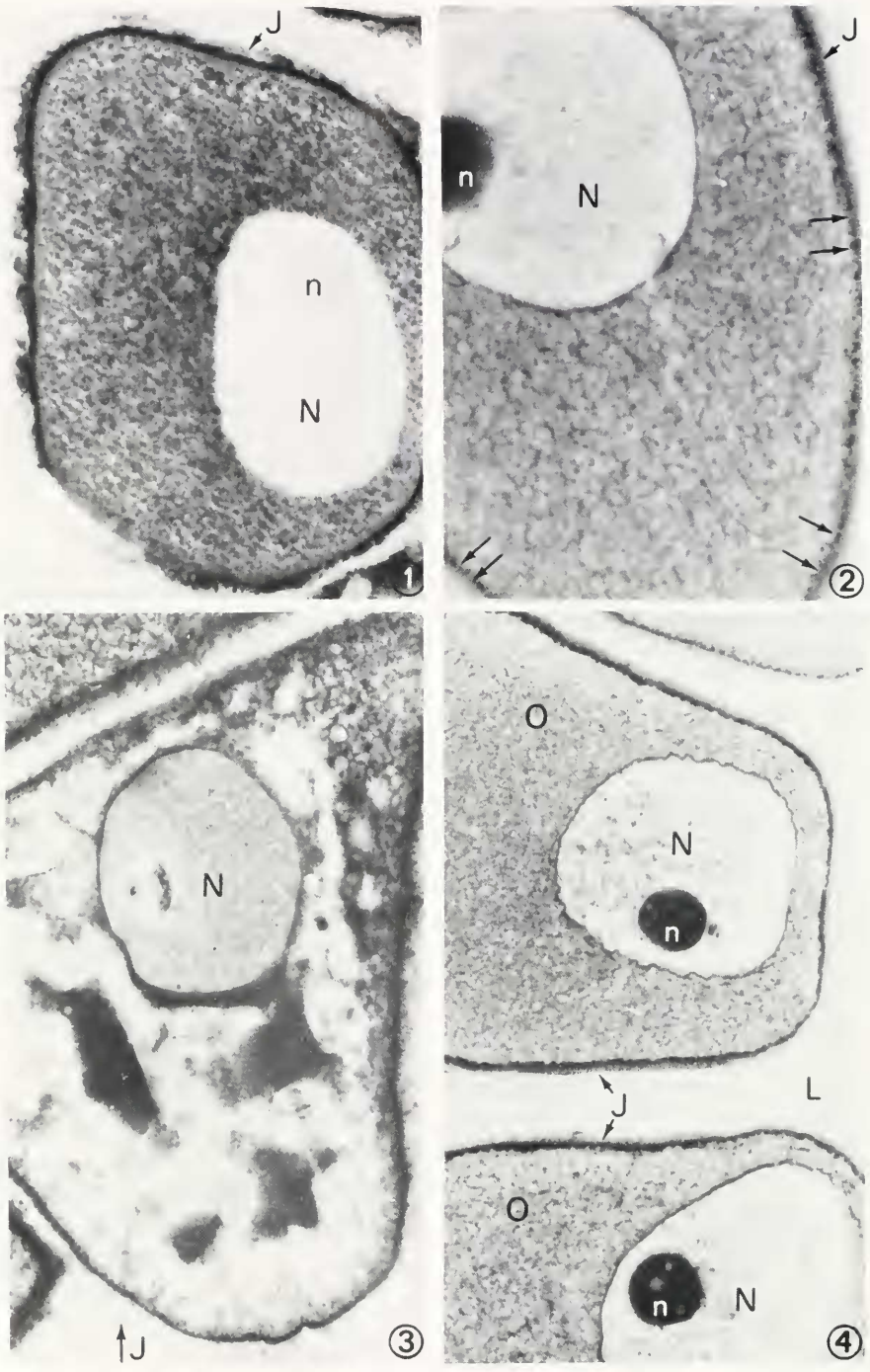

PLATE III: 1. Section of a full-grown oocyte of *Asterias rubens*. PAS/OG staining. ×520. N: Nucleus; n: nucleolus; J: jelly coat.

2. Detail of a full-grown oocyte. Brookes' trichrome staining. ×820. N: Nucleus; n: nucleolus; J: jelly coat. Note the cortical vesicles (arrows).

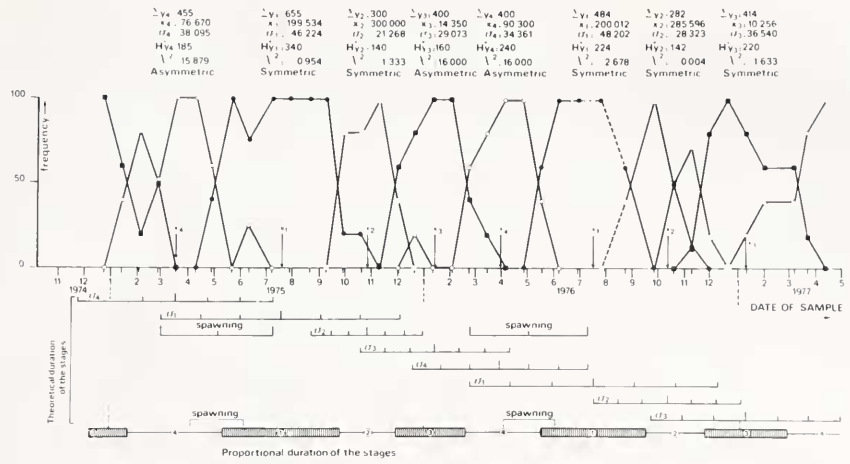

FIGURE 5. Frequency distribution of stages of the annual reproductive cycle of *Asterias rubens* during the period December 1974 to April 1977 (● —— ●: stage 1; □ —— □: stage 2; ■ —— ■: stage 3; ○ —— ○: stage 4); approximation of the theoretical and proportional duration of the stages (see: Tables II and III).

tributions of its substages were not significantly different, and the same applies to the distributions of the substages.

Essential data (the mean and the standard deviation) of the frequency distributions of the stages are summarized in Table II (columns 2 and 3). These data permit an approximation of the theoretical and the proportional duration of each stage, the excentricity of the proportional duration, its corresponding proportional area of each frequency distribution and each tail-probability. These results are given in Table II (columns 4 to 9).

Spawning takes place when the ovaries pass from stage 4 into stage 1. Thus, the spawning period can be defined as the period in which part of the population is in stage 4 and another part in stage 1. The theoretical duration of the spawning period is identical with the theoretical overlap of stage 4 and stage 1. Assuming a normal distribution of spawning animals, the mean and the standard deviation of this distribution can be found, just as the proportional duration of the spawning period, the proportional area and the tail-probability. Data on the spawning period are included also in Table II.

Figure 5 shows the theoretical duration of each stage and the spawning period. The theoretical and proportional duration of each stage, expressed by dates, are given in Table III. As can be noticed from this table, small gaps and overlaps are present between the proportional periods of the stages. Correction gives a continuous sequence of the periods of the stages in time. These "corrected" proportional durations of the stages are presented also in Table III and indicated in Figures 5, 6, and 7.

The proportional duration of stage 1 is the largest one (4 to 4.5 months), followed by stage 4 (3 to 3.5 months), stage 3 (2.5 to 3 months), and stage 2 (2 to 2.5 months).

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3. Section of a degenerating oocyte. Brookes' trichrome staining. ×520. N: Nucleus; J: jelly coat. Note the degenerating cytoplasm of the oocyte.
4. Section of "loose" oocytes. Brookes' trichrome staining. ×520. N: Nucleus; n: nucleolus; J: jelly coat; the oocytes (O) in the ovarian lumen (L) are free from each other. Note the absence of follicle cells and of hemal fluid.

TABLE II

The mean ($\bar{x}$) and the standard deviation (σ) of the frequency distribution of the stages of the annual reproductive cycle of Asterias rubens, *and from these data approximated durations of the stages and the proportional area of the frequency distributions*

Stage	$\bar{x}$	σ	Theoretical duration	Proportional duration	$x - \bar{x}$	$\frac{x - \bar{x}}{\sigma} = u$	Proportional area (one-tailed)	P (one-tailed)
Year 1								
4	76.67	38.10	(2×) 114.3	(2×) 54	54	1.4175	0.4218	0.0782
1	199.53	46.22	277.3	132	66	1.4278	0.4233	0.0767
2	300.00	21.27	127.6	61	30.5	1.4341	0.4242	0.0758
3	14.35	29.07	174.4	83	41.5	1.4274	0.4233	0.0767
4	90.30	34.36	(2×) 103.1	(2×) 49	49	1.4260	0.4231	0.0768
Total of days	378.63		796.7	379				
Spawning	126.50	21.83	131	62	31	1.4198	0.4222	0.0778
Year 2								
4	90.30	34.36	(2×) 103.1	(2×) 44	44	1.2805	0.3998	0.1002
1	200.01	48.20	289.2	123	61.5	1.2759	0.3990	0.1010
2	285.60	28.33	170.0	72	36	1.2708	0.3981	0.1019
3	10.26	36.54	(2×) 109.6	47	47	1.2863	0.4008	0.0992
Total of days	285.96		671.9	286				
Spawning	124.00	23.00	138	59	29.5	1.2826	0.4002	0.0998

The quantitative data were averaged per sample and plotted *versus* time, giving the course of a number of parameters during the annual reproductive cycle. The mean percentage (±SEM) per sample of pear-shaped oocytes and of the PAS-positivity and basophility of the oocytes, the average (±CI) per sample of the pyloric caeca index and of the gonad index, the weighed average per sample of the ten smallest,

TABLE III

The duration of the stages of the annual reproductive cycle of Asterias rubens, *expressed as dates*

Stage	Middle of the period	Theoretical duration		Proportional duration		"Corrected" proportional duration	
		from	to	from	to	from	to
4	03–18	11–23	07–10	01–23	05–11	01–22	05–12
1	07–19	03–02	12–04	05–14	09–23	05–12	09–25
2	10–27	08–24	12–30	09–26	11–26	09–25	11–28
3	01–14	10–19	04–11	12–03	02–24	11–28	02–19
4	03–30	12–18	07–11	02–15	05–13	02–19	05–17
1	07–18	02–24	12–10	05–17	09–17	05–17	09–15
2	10–12	07–19	01–05	09–06	11–16	09–15	11–24
3	01–10	09–23	04–30	11–24	02–26	11–24	02–26
Spawning 1st year	05–06	03–02	07–10	04–05	06–06		
Spawning 2nd year	05–03	02–24	07–11	04–04	06–02		

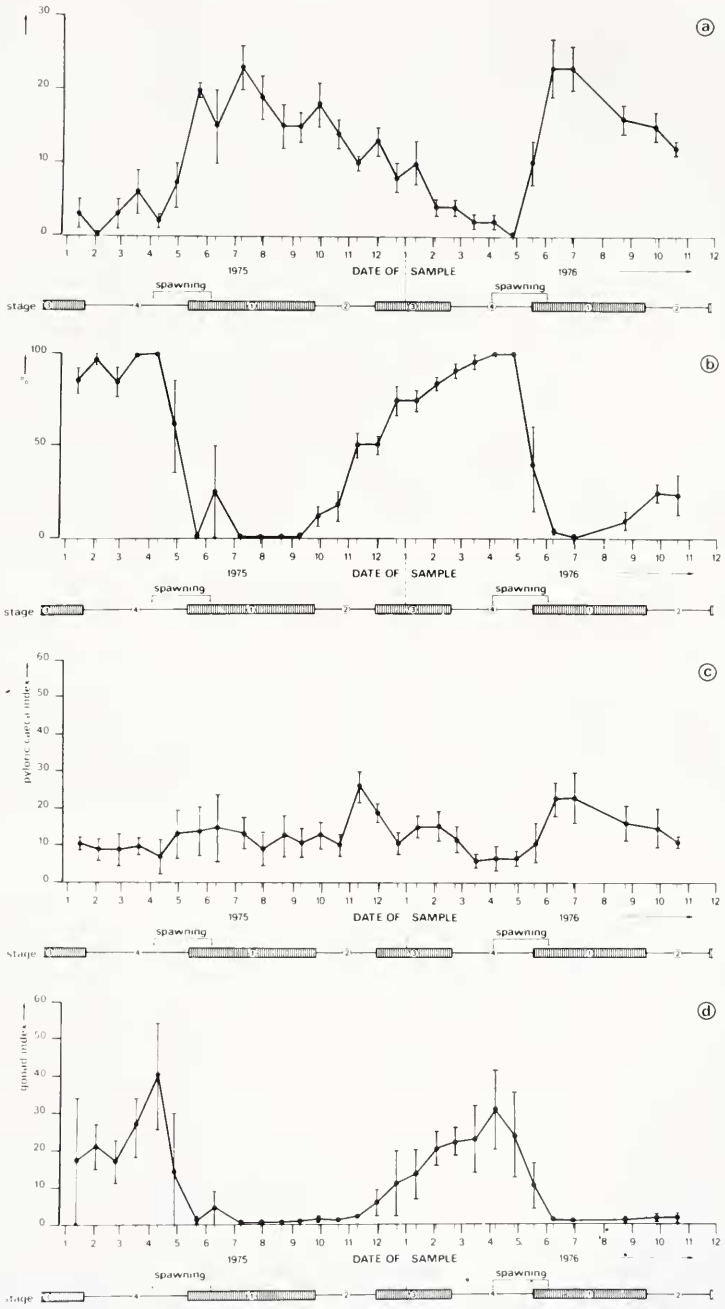

FIGURE 6. a: Mean percentage (±SEM) per sample of pear-shaped oocytes, b: mean percentage (±SEM) per sample of PAS-positivity and basophility of the oocytes, c: average per sample of the pyloric caeca index (±CI) and d: average per sample of the gonad index (±CI) during the annual reproductive cycle of *Asterias rubens*.

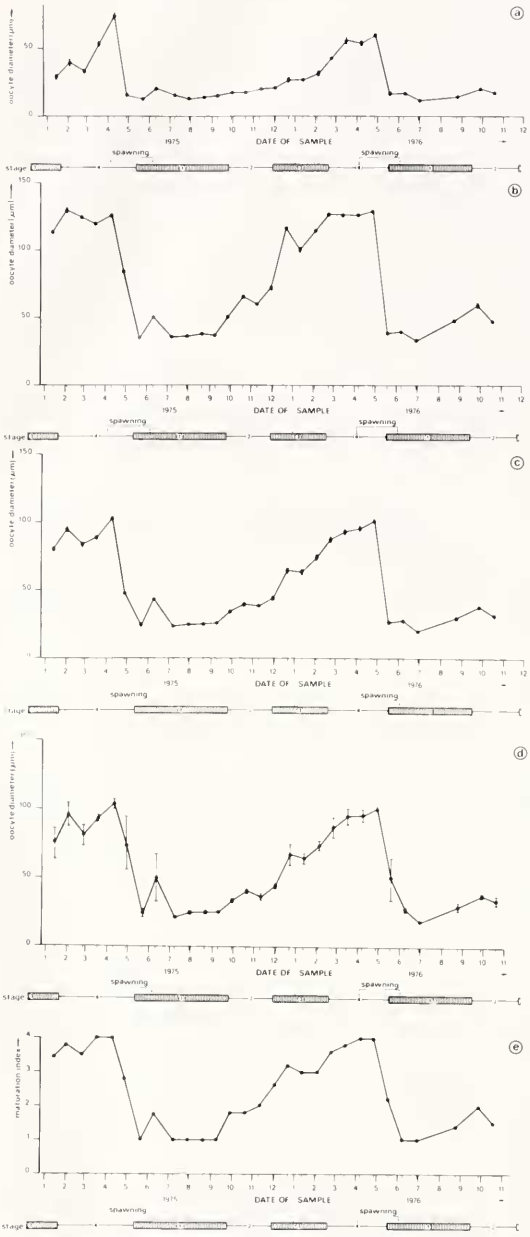

FIGURE 7. a: Weighed average per sample of the ten smallest oocyte diameters (in μm), b: weighed average per sample of the ten largest oocyte diameters (in μm), c: weighed average per sample of hundred oocyte diameters (in μm), d: average per sample of the median oocyte diameter (in μm), and e: maturation index per sample during the annual reproductive cycle of *Asterias rubens*.

of the ten largest, and of the one hundred oocyte diameters, and the mean (±SEM) per sample of the median values during the annual reproductive cycle of *Asterias rubens* are presented in Figures 6 and 7. The course of the maturation index per sample during the annual reproductive cycle is shown in Figure 7e.

The correlations between the quantitatively determined variables (number of animals = 83), expressed as correlation coefficients (r), are presented in a correlation matrix (Table IV). The median, the mean diameter of the one hundred oocytes, of the 10 largest oocytes, and of the 10 smallest oocytes, the percentage of PAS-positive and of basophilic oocytes, and the gonad index all show a significant, strongly positive correlation ($+0.798 < r < +0.998$; $P < 0.005$). These parameters are negatively correlated with the percentage of pear-shaped oocytes, and the correlation is significant ($-0.860 < r < -0.706$; $P < 0.005$). The pyloric caeca index is significantly and negatively correlated with the median, the mean diameter of the one hundred oocytes and of the 10 smallest oocytes, the gonad index ($-0.369 < r < -0.309$; $P < 0.005$), and with the mean diameter of the ten largest oocytes ($r = 0.240$; $0.01 < P < 0.025$); no significant correlation was found between the pyloric caeca index and the percentage of PAS-positive and basophilic oocytes, and the percentage of the pear-shaped oocytes.

Two groups of semi-quantitatively scored parameters can be distinguished. The parameters of group 1 are: dividing oogonia, cell-debris, phagocytozing cells, follicle cells, and invaginations of the hemal system; parameters of group 2 are: hemal fluid, jelly coat, cortical vesicles, degenerating oocytes, and "loose" oocytes. The parameters of group 1 are more or less positively correlated with each other and negatively correlated with the parameters of group 2, which are positively correlated with each other again.

Discussion

The results prove that the reproductive cycle of *Asterias rubens* is an annual cycle, which can be divided into four stages, each comprised of two substages, making a total of eight substages. The subdivision into eight substages is based on the median test, applied to the oocyte diameters of all individual animals, and on the significant differences between the frequency distributions of the oocyte diameters per substage.

Table IV

Correlation matrix for the quantitative data obtained from 83 specimens of Asterias rubens *in different stages of the annual reproductive cycle.*

	1	2	3	4	5	6	7	8
1	1.000	0.998	0.959	0.945	0.877	0.910	−0.817	−0.310
2	0.998	1.000	0.969	0.949	0.878	0.913	−0.822	−0.309
3	0.959	0.969	1.000	0.960	0.826	0.798	−0.819	−0.240*
4	0.945	0.949	0.960	1.000	0.820	0.806	−0.860	−0.159**
5	0.877	0.878	0.826	0.820	1.000	0.833	−0.718	−0.312
6	0.910	0.913	0.798	0.806	0.833	1.000	−0.706	−0.369
7	−0.817	−0.822	−0.819	−0.860	−0.718	−0.706	1.000	0.128**
8	−0.310	−0.309	−0.240*	−0.159**	−0.312	−0.369	0.128**	1.000

[1] The median values.
[2] The mean diameter of the one hundred oocytes.
[3] The mean diameter of the ten largest oocytes.
[4] The percentage of PAS-positive and basophilic oocytes.
[5] The gonad index.
[6] The mean diameter of the ten smallest oocytes.
[7] The percentage of pear-shaped oocytes.
[8] $P < 0.005$. The pyloric caeca index.
* $0.01 < P < 0.025$.
** Not significant.

Also the median values and the averages of the one hundred oocytes support a subdivision into eight substages. This subdivision, based on biometrical data, may be indispensible in studying special aspects, such as vitellogenesis or the influence of endogenous and exogenous factors on the growth of oocytes.

The division of the annual reproductive cycle into four stages is primarily based on the combined semi-quantitative data, but also on a number of quantitatively determined criteria. This division may be useful in studying physiological problems such as energy metabolism and nutrition.

Frequency distributions of oocyte diameters present a characteristic for each stage or substage. Scharf *et al.* pointed out, that normal distribution of variables in morphometry is an exception and recommended to normalize non-normality, if the samples are large ($n > 80$) by McCall transformation. Fichna and Malendowicz (1975) also performed the McCall (1949) transformation according to Scharf *et al.* (1968) in their karyometric studies of the effects of gonadotropin and testosterone on gonadal tissues of the rat.

The McCall (1949) transformation allowed for comparison between the frequency distributions. Since the T-values were calculated from the frequency distribution of oocyte diameters of stage 3, this stage is the reference for each other stage or substage. By applying these calculated T-values to corresponding oocyte diameters of any other experimental group of animals, it is possible to compare the frequency distribution of oocyte diameters of this group with those of the stages and substages of the annual reproductive cycle presented in this study. Schoenmakers *et al.* (1981a) reported such a comparison with control and treated animals in their study on the effects of oestradiol-17β on the ovaries of the starfish *Asterias rubens*.

The frequency distributions of the stages also support a division into four stages since the frequency distributions of the substages of any one stage do not significantly differ from each other, nor from that of that stage, meaning that the substages are completely synchronic. The theoretical duration of the stages shows overlaps. Thus, parts of the four stages are synchronic. By approximating the proportional durations of the stages, a chronological sequence of the stages during the annual reproductive cycle was found. These results make it possible to predict, with a one side tail-probability of 8–10%, the period of the year in which 80–85% of the starfish population is in any one stage of the annual reproductive cycle. The duration of the stages and the time when a stage begins and ends probably will vary slightly from year to year. These variations are probably affected by the availability and amount of food, and by climatic conditions, especially temperature. Since the variations were found to be not significant, it may be concluded that, within the mentioned limits, the proportional durations of the stages and their chronological sequence in the annual reproductive cycle appear to be reproducible from year to year.

In the present study a number of variables have been studied quantitatively. By measuring the diameters of one hundred oocytes in representative areas of the ovaries, information was obtained, which could be processed for different purposes. Statistical analysis of the data indicated the presence or absence of differences between individual animals and made it possible to distinguish eight substages. The mentioned data formed the basic material for the frequency distributions of oocyte diameters, thus presenting a characteristic for each substage. Also the median values, the averages of the one hundred oocytes, of the ten smallest, and of the ten largest oocytes were calculated. The first two variables, both giving a characteristic of the oocytes in general, support the subdivision into eight substages. The last two variables show a more differentiated picture of the actual condition, in other words the oocytes in each animal are seldom simultaneously in the same phase of oogenesis.

The averages of the ten smallest oocytes, which may be less than 20 μm even in stage 3, demonstrate that only at the end of the reproductive cycle are all oocytes in the same phase of oogenesis. Therefore, it can be concluded that the oocyte diameter is a reliable parameter for delineating the reproductive cycle and oogenesis.

Other variables, based on the oocytes proper, are typical for the actual condition of the growing oocytes. For instance, PAS-positivity and basophilia, which are absent in stage 1, determine the course of vitellogenesis; the jelly coat, cortical vesicles, and "loose" oocytes indicate the maturation of oocytes. Moreover, other cells or features, related to the development of oocytes, *e.g.*, the presence of follicle cells, invaginations of the hemal system, and the hemal fluid, are characteristic for the stages of the annual reproductive cycle. The gonad index and most of the other quantitative data show a significant and negative correlation with the pyloric caeca index. It may be concluded that substances needed for vitellogenesis and growth of the oocytes are transported from the pyloric caeca to the ovaries.

This detailed description of the annual reproductive cycle of *Asterias rubens*, including a clearly defined subdivision in stages and substages, required the analysis of a complex of variables. However, a broad outline of the annual reproductive cycle can be carried out with a few variables only, since correlations were found between the quantitatively determined variables and between the semi-quantitative data. Indeed, one variable (for example, the gonad index) may be sufficient.

ACKNOWLEDGMENTS

The authors wish to thank Prof. Dr. P. G. W. J. van Oordt for his critical comments. Appreciation is expressed to Prof. Dr. A. Lindenmayer and Prof. Dr. G. Leppink for their advices on statistics. Thanks are also due to Ms. Drs. M. Rapis and Mr. J. Quaadvliet for their technical assistance.

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